FORM PTO-1390 (REV 10-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

# TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 205,360

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 10/018451

| INTERNATIONAL APPLICATION NO. | INTERNATIONAL FILING DATE | PRIORITY DATE CLAIMED |
|---|---|---|
| PCT/EP00/03586 | April 20, 2000 | April 22, 1999 |

TITLE OF INVENTION: INACTIVATED MICROORGANISMS COMPRISING SUBSTANCES HAVING PHARMACOLOGICAL ACTIVITY

APPLICANT(S) FOR DO/EO/US: Ernst Bernhard GRABITZ

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). - unsigned
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

**Items 11 to 16 below concern document(s) or information included:**

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information:

"EXPRESS MAIL" label no.: EF174014781US
Date of Deposit: October 22, 2001
This correspondence is being Deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR § 1.10 on the date indicated above and addressed to: Assistant Commissioner for Patents Washington, D.C. 20231.

531 Rec'd PCT/PT 22 OCT 2001

| U.S. APPLICATION NO. (if known, see 37 CFR 1.5) | INTERNATIONAL APPLICATION NO. | ATTORNEY'S DOCKET NUMBER |
|---|---|---|
| 10/019451 | PCT/EP00/03586 | 205.360 |

17. ☒ The following fees are submitted:

**BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):**

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... **$1000.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... **$860.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... **$710.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... **$690.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... **$100.00**

| CALCULATIONS PTO USE ONLY | |
|---|---|
| $ 890.00 | |
| **ENTER APPROPRIATE BASIC FEE AMOUNT =** | $ 890.00 |
| Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)). | $ |

| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE | |
|---|---|---|---|---|
| Total claims | 24 - 20 = | 4 | X $18.00 | $ 72.00 |
| Independent claims | - 3 = | | X $80.00 | $ |
| MULTIPLE DEPENDENT CLAIM(S) (if applicable) | | | + $270.00 | $ |
| **TOTAL OF ABOVE CALCULATIONS =** | | | | $ 962.00 |
| ☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. | | | | $ 481.00 |
| **SUBTOTAL =** | | | | $ 481.00 |
| Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). + | | | | $ |
| **TOTAL NATIONAL FEE =** | | | | $ 481.00 |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property + | | | | $ |
| **TOTAL FEES ENCLOSED =** | | | | $ 481.00 |
| | | | Amount to be refunded: | $ |
| | | | charged: | $ |

a. ☒ A check in the amount of $ 481.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 01-0035. A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:

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Oct.22, 2001
SIGNATURE

Jay S. Cinamon
NAME

24,156
REGISTRATION NUMBER

PATENT DOCKET 205,360

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | |
|---|---|---|
| APPLICANT: | GRABITZ | EXAMINER: |
| SERIAL NO.: | Not Yet Assigned | ART UNIT.: |
| FILED: | Herewith | |
| TITLE: | INACTIVATED MICROORGANISMS COMPRISING SUBSTANCES HAVING PHARMACOLOGICAL ACTIVITY | |

DATE: October 22, 2001

PRE-EXAMINATION AMENDMENT

Hon. Commissioner of
Patents and Trademarks
Washington, D.C. 20231
SIR:

STATEMENT OF FILING BY EXPRESS MAIL 37 C.F.R. § 1.10

This correspondence is being deposited with the United States Postal Service on October 22, 2001 in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EF 174 014 781 US addressed to the Honorable Commissioner for Patents, Washington, D.C. 20231.

Please amend the application filed on even date herewith prior to proceeding with its examination.

IN THE CLAIMS

Please amend claims 8, 9, 13-16, and 18-21, as follows:

8. (Amended) Inactivated microorganisms according to claim 1, wherein said microorganism is Saccharomyces cerevisiae.

9. (Amended) Food compositions wherein the inactivated microorganisms according to claim 1 are used.

13. (Amended) Use of one or more inactivated micoorganisms according to claim 1 in human or animal alimentation.

14. (Amended) Use of the microorganisms according to claim 1 as components of feeding or premixes in zootechnics.

15. (Amended) Use of the microorganisms according to claim 10 for the feeding of fish.

16. (Amended) Process for the preparation of inactivated microorganisms containing one or more soluble and/or solubilizable substances having pharmacological activity and/or nutritional substances having pharmacological activity, according to claim 1 comprising the following steps:

(i) drawing out the endocellular mass of a suitable microorganism by means of hypertonic treatment, separation of the drawn out endocellular mass and recovery of the empty microorganisms;

(ii) optional inactivation of the microorganism obtained in

Step i) chemically or physically, leaving the external membrane of the microorganism unaltered; and

(iii) intracellular loading of one or more soluble and/or solubilizable substances having pharmacological activity and/or nutritional substances having pharmacological activity, into the inactivated microorganism obtained in Step i) or Step ii), is obtained by means of hypo- and/or iso-tonic treatment.

18. (Amended) Process according to claim 16 wherein step iii) is followed by treatment of the microorganisms with a fixative or a disinfectant agent.

19. (Amended) Process for the preparation of inactivated microorganisms according to claim 1 which comprises the following steps:

1) inactivating the microorganism by thermal treatment, at 60-65°C for 30-120 min;

2) resuspending the inactivated cells of the microorganism in an isotonic medium comprising the active principle to be incorporated;

3) stirring the suspension for 48-72 hours;

4) centrifuging the suspension;

5) optional buffering and/or fixation is carried out using formalin and/or glutaraldehyde.

20. (Amended) Process according to claim 16 wherein the hypertonic treatment in step i) is obtained by incubation of a hypertonic solution comprising:

NaCl in concentrations higher than 0.2 M; and

optionally sodium citrate which is between 0.03 to 0.1 M.

21. (Amended) Process according to claim 16, wherein said hypotonic treatment in step iii) is obtained by means of a hypotonic solution comprising:

NaCl in concentrations lower than 0.12 M; and

optionally sodium citrate in concentrations lower than 0.025 M.

REMARKS

It is respectfully requested that the examination of this application proceed on the basis of the amendatory action taken herein and that this amendment be entered prior to calculating the filing fee and according the application a filing date.

Attached hereto is a Marked-Up Version of the Changes Made to the claims by the current Pre-Examination Amendment. The attached page is captioned "Marked-Up Version to Show Changes Made".

The issuance of a Notice of Allowance is respectfully solicited.

Please charge any fees which may be due and which have not been submitted herewith to our Deposit Account No. 01-0035.

Respectfully submitted,

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Docket No.: 205.360

MARKED-UP VERSION TO SHOW CHANGES MADE BY AMENDMENT

Claims 8, 9, 13-16, and 18-21 have been amended as follows:

8. (Amended) Inactivated microorganisms according to claim~~s~~ 1 ~~-~~7, wherein said microorganism is Saccharomyces cerevisiae.

9. (Amended) Food compositions ~~characterized in that~~ wherein the inactivated microorganisms according to claim~~s~~ 1 ~~-~~8 are used.

13. (Amended) Use of one or more inactivated micoorganisms according to claim~~s~~ 1 ~~-~~12 in human or animal alimentation.

14. (Amended) Use of the microorganisms according to claim~~s~~ 1 ~~-~~12 as components of feeding or premixes in zootechnics.

15. (Amended) Use of the microorganisms according to claim~~s~~ 10 ~~-~~12 for the feeding of fish.

16. (Amended) Process for the preparation of inactivated microorganisms containing one or more soluble and/or solubilizable substances having pharmacological activity and/or nutritional substances having pharmacological activity, according to claim~~s~~ 1 ~~-~~12 comprising the following steps:

(i) drawing out the endocellular mass of a suitable microorganism by means of hypertonic treatment, separation of the drawn out endocellular mass and recovery of the empty microorganisms;

(ii) optional inactivation of the microorganism obtained in

Step i) chemically or physically, leaving the external membrane of the microorganism unaltered; and

(iii) intracellular loading of one or more soluble and/or solubilizable substances having pharmacological activity and/or nutritional substances having pharmacological activity, into the inactivated microorganism obtained in Step i) or Step ii), is obtained by means of hypo- and/or iso-tonic treatment.

18. (Amended) Process according to claims 16 ~~and 17 characterized in that~~ wherein step iii) is followed by treatment of the microorganisms with a fixative or a disinfectant agent.

19. (Amended) Process for the preparation of inactivated microorganisms ~~described in~~ according to claim~~s~~ 1 ~~-12~~ which comprises the following steps:

1) inactivating the microorganism ~~is inactivated~~ by thermal treatment, at 60-65°C for 30-120 min;

2) resuspending the inactivated cells of the microorganism ~~are resuspended~~ in an isotonic medium comprising the active principle to be incorporated;

3) stirring the suspension ~~is left stirring~~ for 48-72 hours;

4) centrifuging the suspension ~~is centrifuged~~;

5) optional buffering and/or fixation is carried out using formalin and/or glutaraldehyde.

20. (Amended) Process according to claim~~s~~ 16 ~~and 17 characterized in that~~ wherein the hypertonic treatment in step i) is obtained by incubation of a hypertonic solution comprising:

NaCl in concentrations higher than 0.2 M; and

optionally sodium citrate which is between 0.03 to 0.1 M.

21. (Amended) Process according to claims 16 ~~and 17~~, ~~in which~~ wherein said hypotonic treatment in step iii) is obtained by means of a hypotonic solution comprising:

NaCl in concentrations lower than 0.12 M; and

optionally sodium citrate in concentrations lower than 0.025 M.

# INACTIVATED MICROORGANISMS COMPRISING SOLUBLE AND/OR SOLUBILIZABLE SUBSTANCES HAVING PHARMACOLOGICAL ACTIVITY AND/OR NUTRITIONAL SUBSTANCES HAVING PHARMACOLOGICAL ACTIVITY, THE PROCESS FOR THEIR PREPARATION AND THEIR USES THEREOF

## Scope of Invention

The present invention is related to inactivated microorganisms comprising soluble and/or solubilizable substances having pharmacological activity and/or nutritional substances having pharmacological activity, which are absorbed by the systemic circulation of the human or animal organism. A process for their preparation and their uses thereof are described.

## State of the art

The compounds generally known as soluble and/or solubilizable substances having pharmacological activity and nutritional substances having pharmacological activity refer to active agents and/or substances which, in order to perform their activity, need to penetrate into the systemic circulation of the animal or human organism.

Soluble and/or solubilizable substances having pharmacological activity comprise, for example, drugs and vaccines.

The nutritional substances having pharmacological activity comprise, for example vitamins, amino acids, and food integrators in general, etc.

Pharmacologically active substances and pharmacologically active nutritional substances that involve oral administration need to reach in an intact and active form at the level of the intestine and, in order to be correctly processed, need to penetrate into the systemic circulation and perform their pharmacological or nutritive function effectively.

The oral administration of drugs presents various serious problems, such as emesis resulting from irritation of the gastro-intestinal mucosa, destruction of certain drugs by digestive enzymes or, on account of the low gastric pH, irregularity in absorption or in peristalsis in the presence of food or of other medicinal preparations. Furthermore, drugs in the gastro-intestinal tract may be metabolized by enzymes of the mucosa, by the intestinal flora or by the liver,

before reaching the general circulation.

In particular, the wide pH range that is found in the gastro-intestinal tract may affect the rate of absorption, altering the relative concentrations of ionized forms and non-ionized forms.

Consequently, drugs and nutritionally active agents administered by oral route present the serious problem that during their passage through the gastric tract they may lose totally or partially their pharmacological efficacy and/or nutritional activity or their bio-availability in the circulation may be impaired.

A further problem is the thermostability of the active agents. In fact, a procedure of administration of said active agents consists in mixing them with food to facilitate their uptake by humans or by animals. Many food preparations require, at the moment of their administration, heating (in some cases also boiling), and this consequently involves the denaturation or thermo-inactivation of those active agents that are thermolabile.

In addition, drugs and nutrients endowed with marked characteristics of hydrophilicity (solubility in water), such as vitamins C and B12, many antibiotics and antibacterial agents, cannot be administered to fishes on account of their solubility in the aqueous medium, even when mixed to nutrients.

Therefore the technical problem in the current state of the art consists in supplying to the systemic tract of the animal or human organism active compounds, in the form of pharmacologically and/or nutritionally active agents ingested by oral route, which will maintain their own pharmaceutical or nutritive capabilities in an unaltered form, so avoiding the problems due to their passage through the gastric tract. A further problem is the prevention of the alteration of these active agents during preparation.

The patent application EP 0 899326 describes the use of a yeast for the transport and release of exogenous digestive enzymes in the stomach. The addition of exogenous enzymes in the stomach is important, for preventive purposes, in the case of animals undergoing intensive feeding or pharmacological treatments, in that they digest food and drugs, inactivating the latter. The microorganisms thus obtained, are mixed in the food compositions and start pre-digestion in the gastric tract.

However the method described in EP 0 899326 refers exclusively to a way to protect digestive enzymes (i.e., substances that are not pharmacologically or nutritionally active) and to release them in the stomach and to their use as adjuvants during intensive feeding or intensive pharmacological treatments.

The present invention intends to solve the problem of delivery of active agents (pharmacologically and nutritionally active agents) in a protected form up to the intestine, i.e., to the site of penetration into the systemic circulation and preventing, up to that moment, any active system to be modified, inactivated or even partially digested by digestive enzymes and by the gastric environment.

**Summary of the invention**

The present inventors have solved these technical problems and have obtained inactivated microorganisms comprising soluble and/or solubilizable substances with pharmacological activity (pharmacologically active agents) and/or nutritional substances having pharmacological activity (nutritionally active agents). Said microorganisms are thus modified and used to protect said active agents during their passage through the stomach into the intestine where said active agents are released and absorbed by the systemic circulation of animals or humans.

Hence, according to a first embodiment, the present invention refers to inactivated microorganisms comprising soluble and/or solubilizable substances and/or nutritional substances with pharmacological activity, such as medicinal preparations or vaccines or nutritionally active agents in general such as vitamins, amino acids, etc.

According to a further embodiment, the invention refers to a process for the preparation of inactivated microorganisms according to the invention comprising the steps of:

i) drawing out the endocellular mass of a suitable microorganism by means of hypertonic treatment;

ii) optional chemical or physical inactivation of the microorganism obtained in Step i), which leaves the external membrane of the microorganism unaltered;

iii) intracellular loading of one or more soluble and/or solubilizable substances having pharmacological activity and/or nutritional substances with pharmacological activity into the inactivated microorganism obtained in Step i) or Step ii) by means

of hypo- or isotonic treatment.

In the present description isotonic solution means a solution having the same osmotic pressure as the fluid phase of a cell or a tissue and corresponding to the osmotic pressure of a 0.9% NaCl solution (physiologic saline)). Hypertonic and hypotonic solutions have meanings related to this definition (i.e. > or < than a 0.9% NaCl solution, respectively).

According to a further embodiment, in Step i) the microorganic endocellular mass is drawn out by treatment with a hypertonic solution, where the hypertonicity is provided by the pharmacologically active principle itself.

According to yet a further embodiment, the invention refers to a process in which the introduction of the active principle into the microorganism is carried out in the presence of an isotonic solution in Step iii) of the process.

According to a further embodiment, the invention refers to the use of such inactivated and loaded microorganisms in human or animal alimentation, or also as components of feeds or premixes.

The present invention, in addition, refers to a composition comprising an effective quantity of one or more types of organisms prepared according to the invention.

**Detailed description of the invention**

The present invention enables administration, by oral route, of soluble and/or solubilizable substances having pharmacological activity and/or nutritional substances having pharmacological activity, incorporated within the cell walls of inactivated, i.e. "killed" microorganisms, capable of protecting their characteristics of structure and activity. The active agents are thus protected on arrival at the level of the intestine and, once released, enter the systemic circulation where they perform their activity.

*Microorganisms*

The microorganisms used in the process according to the present invention must be microorganisms with a good resistance to chemical and chemico-physical stress. The said microorganisms may be selected also on the basis of the characteristics of affinity and tolerability in regard to the host organisms.

Among known and commercially available microorganisms, the yeast *Saccharomyces cerevisiae*, for example, is preferred because of its structural

resistance to chemical and chemico-physical agents, being able to reach the intestine unaltered, and to release there the active agents where they are taken into the systemic circulation of the animal or human organism.

Some of the microorganisms that can be used are, for example, those which normally are present in the intestinal microflora, such as *Bacillus subtilis* and *Lactobacillus sp.*, which are also readily available on the market.

It is also possible to use the so-called "vulgar" microorganisms, isolated from human faecal material, belonging to the so-called "good" intestinal flora, or from ruminal, enteric or faecal material of the "good" intestinal flora and of other sources of the various animal breeding species. Isolation is carried out using methodologies known in the state of the art, by growing the microflora in nutrient culture and subsequently selecting the colonies on the basis of their morphological and taxonomic characteristics. The characterization of the isolated strains takes place according to commonly used methodologies, for example from the analysis of the Gram colouring, metabolic behaviour, production of characteristic chemical products and the nutritive characteristics.

Other microorganisms that may be advantageously used in the process of the invention, together with some of their sources, appear in "Biochemical Engineering and Biotechnology Handbook", B. Atkinson and F. Mavituna Eds., 1991, MacMillan Publ., Chaps. 6.1, 6.7 and 9.5.

*Active agents*

The soluble and/or solubilizable substances having pharmacological activity and the nutritional substances having pharmacological activity according to the invention are defined as follows.

The soluble and/or solubilizable substances having pharmacological activity according to the invention comprise, for example, all pharmaceutical substances, antibiotic, antibacterial, hormones, anti-inflammatory, antiviral, antifungal, antiparassitic agents and other substances, and vaccines provided that they are soluble and/or solubilizable in an aqueous medium.

The nutritional substances having pharmacological activity comprise, for example, vitamins such as ascorbic acid, folic acid, cyanocobalamin, thiamine or α-tocopherol, amino acids, or oligoelements such as cofactors and minerals such as

zinc or cobalt, food integrators of various kinds, active principles of vegetable origin and/or the substances known as «nutriceuticals», for example, bioflavonoids such as sodium quercetin, catechin, isocatechin, flavans, cyanins, polyphenols, aliphatic polyalcohols, resveratrol, hyperic acid, rutinoids, etc.

*Process of preparation*

The preparation of inactivated microorganisms comprising soluble and/or solubilizable substances and/or nutritional substances having pharmacological activity according to the invention comprises the steps of:

i) drawing out the endocellular mass of an appropriate microorganism by means of hypertonic treatment;

ii) optional chemical or physical inactivation of the microorganism obtained in Step i), which leaves the external membrane of the microorganism unaltered;

iii) intracellular loading of one or more soluble and/or solubilizable substances having pharmacological activity and/or nutritional substances having pharmacological activity in the inactivated microorganism obtained in Step i) or Step ii) by means of hypo- or isotonic treatment.

Before the drawing out step (Step i), microorganisms can be grown in appropriate fermenters, according to the methods and conditions known in the state of the art, separating the microrganic mass from the culture medium at the end of the growth phase, by means of filtration or centrifugation. An example of the operating conditions is provided in "Biochemical Engineering and Biotechnology Handbook", B. Atkinson and F. Mavituna Eds., 1991, MacMillan Publ., Chap. 6.7.

In Step i) the endocellular mass is squeezed out of the microrganic cell walls by means of a hypertonic treatment.

Step i) is performed by resuspension of the microrganic mass in a hypertonic aqueous solution. Hypertonicity can also be achieved by gradually increasing the salt concentration at the hypertonic endpoint.

The hypertonic aqueous solution may comprise:

- NaCl in concentrations higher than 0.2 M, preferably 1.0 M;
- optionally the citrate ion, which contributes to the enlargement of the membrane pores; although not strictly indispensable, it is preferred that sodium citrate is present in concentrations ranging from 0.03 and 0.1 M, preferably 0.05 M.

Preferably, the aforesaid hypertonic solution comprises NaCl 1.0 M and sodium citrate 0.05 M.

An appropriate antibacterial and/or fixative agent may be possibly added, such as polyphosphates or para-benzoates, formaldehyde or pentaraldehyde.

The suspension obtained is stirred at a temperature ranging from 2 to 40°C, preferably at 25°C, for a period ranging from 2 hours to 4 days, preferably 16 hours, to complete emptying of the microorganisms in such a way that the endocellular content is extruded into the hypertonic medium.

The so treated microorganisms are reduced to the cell walls alone and are smaller than the alive microorganisms; they presents enlarged membrane pores. Empty cell walls may be separated from the endocellular mass by means of techniques known in the state of the art, such as filtration or centrifugation.

The control to ensure that the endocellular mass has been removed to leave just the cell walls may be conveniently performed by morphological microscopic evaluation of the cell walls, which must appear smaller and shrivelled.

The suspension medium (i.e. the hypertonic buffer and the squeezed out endocellular content) can be separated from the microorganisms cell walls, by centrifugation at 2.000-12.000 r.p.m., preferably 4.500 r.p.m (corresponding to about 4600 R.C.F.).

The centrifugates may be optionally washed. In particular, aliquots are used in analytical control to evaluate the quantity of incorporated active agent.

In Step ii), emptied microorganisms may be furtherly inactivated, i.e. killed, by means of appropriate chemical or physical treatment. This treatment may not be required for certain microorganisms, because the hypertonic treatment of Step i) may be sufficient for inactivation. Consequently, Step ii) is carried out only after checking the viability of microorganisms after Step i).

Among the chemical methods preferably used for microorganisms having cells walls that are easily heat-degradable, it is possible to use treatment with disinfectant or fixing substances, such as formalin (formaldehyde) or glutaraldehyde, at concentrations of 0.05-0.2 mg/l, for a time ranging from 5 minutes to 12 hours, preferably at the concentration of 0.1 mg/l, for 6-8 hours.

The chemical inactivation can take place also during the Step i), by adding the

above-mentioned disinfectant and/or fixing substances to the hypertonic solution.

Physical treatment preferably comprises exposure to UV radiation and thermal inactivation by heating the suspension obtained from Step i) to a temperature of between 55 and 65°C, preferably 60°C, and finally isolating the inactivated microorganisms by concentration and filtration.

This treatment may be carried out also at the beginning of Step iii) of intracellular loading in hypo- or isotonic medium. In this case, emptied microorganisms obtained from Step i) are resuspended in one part of the hypo- or isotonic medium containing the substances to be re-incorporated, and the mixture thus obtained is then heated in the conditions described above. After cooling to 2-8°C, preferably at 4°C, the remaining part of the hypo- or isotonic solution is added, and the step of intracellular loading is then carried out, as described in Step iii).

Inactivation conditions must be such as not to cause alterations in the wall structure of the microorganism, and must be chosen according to the characteristics of resistance of the microorganism itself. Control of the degree of inactivation of the cells of the microorganism at the end of Step ii) is carried out by culture tests on Platecount agar, as here briefly described: a small aliquot of treated micro-organic cells are resuspended in an aliquot of the previously extracted solution, dialysis is then performed and the isotonicity of the solution is re-established, whenever necessary. When the cells have re-acquired their original shape, approximately 1 g or 1 ml of the suspension is inoculated into a selective culture medium (approximately 15-25 ml of culture broth) and incubated (at approx. 37°C for bacteria; at approx. 25°C in the case of mycetes) for 36-48 hours. Since this first phase may not be sufficient to recover viable microorganisms, seeding may be repeated by further inocula (preferably 3), using the culture broth from the step i). Possible growth processes are checked by analysis of the concentration of the microrganic colonies present by turbidimetric analyses or microscope observation (on a slide after colouring), or by colony count.

In Step iii) one or more of the active agents according to the invention are loaded into the inactivated empty microrganic cells, by incubating said cells in a hypo- or isotonic aqueous solution containing the pharmacologically and/or nutritionally

active agents, under gentle stirring, for a time sufficient for the uptake of the active agent into the cells, preferably for a period ranging from 4 hours to 4 days, preferably 16 hours, at a temperature of between 2 and 40°C, preferably at 25°C.

The hypotonic aqueous solution preferably comprises:

- NaCl in concentrations lower than 0.12 M;
- optionally sodium citrate in concentrations lower than 0.025 M;

Preferably, the above-mentioned hypotonic solution comprises NaCl 0.05 M and sodium citrate 0.005 M.

Optionally, a suitable antibacterial agent, such as polyphosphates or para-benzoates may be added.

The isotonic aqueous solution is preferably a 0.9% (w/v) NaCl solution, optionally comprising also low concentrations of sodium citrate ranging from 0.01 to 0.05 M.

Preferably, the isotonic solution according to the invention is a 0.9% NaCl solution comprising 0.025 M sodium citrate.

According to the present invention, the intracellular loading is performed by incubation of the cells in the hypotonic solution comprising the active agent/s which is then absorbed into the microorganisms. NaCl is then continuously added to the solution until isotonicity is reached.

With this treatment, microorganisms re-acquire their original shape. Control of absorption (microorganism loading) can be carried out also by measuring the amount of active agent still present in the aqueous suspension.

According to a further embodiment, when intracellular loading is performed by incubation in isotonic solution, cells are suspended in the isotonic medium comprising the active (agent) or principles; after such incubation emptied cells re-acquire their original shape. Control of absorption is carried out by checking the amount of active agent still present in the isotonic aqueous suspension.

According to another embodiment, the emptying in Step i) is carried out by incubating the cells in a hypertonic solution of the active agent itself. In this case, a pharmacologically or nutritionally active agent according to the invention is prepared in a hypertonic form. The hypertonic solution of the active agent is added, optionally in the presence of sodium citrate, to the solution comprising microorganisms, and emptying-out of the endocellular component from the cell

(microorganism) is obtained.

At this point, after optional chemico-physical inactivation of the microorganisms (Step ii), the hypertonic solution is diluted to values of hypo- or isotonicity (Step iii), thus allowing the active agent to enter into the cell and the latter to recover its shape.

The advantage of this alternative procedure lies in that all the operations are carried out in one and the same medium and container.

The efficiency of loading is good and equivalent according to both embodiments.

According to a further embodiment, introduction of the active agent into the microorganism is performed in the presence of an isotonic solution, according to the following steps:

1) the microorganism is inactivated by thermal route, for example at 60-65 °C for 30-120 min, preferably at 65°C for 30 min;

2) inactivated cells are re-suspended in NaCl isotonic medium containing the active principle to be incorporated;

3) the suspension is left under stirring for 48-72 hours in the same conditions as set out for the other examples; and

4) the suspension is centrifuged, as previously described.

Once loading of the active agent into the cell is completed, according to the processes of the present invention, it is possible to add a buffer solution containing a stabilizer for the cells (which acts by stabilizing the membrane pores), or a fixing agent and hence to stop or limit leakage of the active agent from the cell.

The buffer used may be a suspension or a solution comprising, for example, a fixing agent such as formaldehyde or glutaraldehyde.

Alternatively, stabilization of the cell membrane pores may be carried out by thermal treatment (at 60-65° C for 30-120 min, preferably 60 min).

Finally, the microorganisms obtained from Step iii) may be concentrated, preferably by filtration, to a small volume of hypo- or isotonic medium, or also may be used directly for administration or for the preparation of foodstuff compositions, or also may be separated by centrifugation from the medium.

Alternatively, the microorganisms can be separated from the culture medium by filtration or centrifugation, and the supernatant recovered. The supernatant is then

concentrated, and in case the active agent is a protein, it can be recovered by saline precipitation with ammonium sulphate, as described by Robert K. Scopes in "Protein Purification, Principles and Practice", Chap. 3, Pages 39-52, Springer-Verlag New York Inc., 1982. The protein precipitate can then be separated by filtration, re-suspended in water and dialysed against water with membranes of suitable molecular cut-off (i.e. 2 000/5 000 daltons), for 48 hours, operating at cold temperatures.

The loaded microorganisms are then ready for use in foodstuff preparation or in animal feed. At the intestinal level, the microorganism cell walls are lysed and the active agents incorporated therein are then released. The protected active agents maintain their original properties unaltered, as they do not get in contact with intestinal de-activating factors.

A further object of the present invention is the use of the above-mentioned inactivated microorganisms containing active agents, in the field of foodstuff for human consumption and/or for animal feeding, preferably in zootechnics, namely in the rearing of livestock.

Finally, a further object of the present invention is represented by food compositions, for both human and animal alimentation, comprising inactivated microorganisms enriched with one or different active agents prepared according to the present invention, in association with conventional foodstuffs or animal feed, and possibly in the presence of appropriate excipients and/or diluents. The quantity of microorganisms to be administered varies according to the animal species treated, the diet followed, the general state of health of the animal, and the conditions of rearing, and preferably ranges from 0.1 to 20 g/kg of animal feed, and from 0.1 to 5 g/day for humans. For poultry, the microorganisms of the invention are preferably administered in a quantity of between 30 and 70 mg/day.

The above-mentioned compositions are preferably used in the livestock-rearing sector, in the form of premixes or feed. In fact, the cell walls of the microorganisms are able to protect the characteristics of structure and activity of the active agents contained therein, both in the phase of preparation of the mixture and during its passage through the gastric tract.

It has been found that the active agents inserted into the microorganisms

according to the invention are in particular, stable to changes in temperature, and consequently they are particularly suitable for preparations that require heating of the composition. This is important, for example, when the active agent is mixed with foodstuff or animal feed, or when the food mixture is heated or boiled before ingestion by humans or by animals.

In particular, as described in Example 5, it has been found that the ascorbic acid contained in the microorganism prepared according to the invention is thermostable even at 120°C, whereas it is known from the literature that ascorbic acid as such is easily denatured at much lower temperatures.

*EXPERIMENTAL PART*

The present invention will now be described with reference to particular embodiments in the examples that follow.

**Example 1: Yeasts modified by incorporation of ascorbic acid**

In this assay, yeasts are partially emptied with a hypertonic NaCl solution and loaded with an isotonic ascorbic acid solution.

Step I) – 30 g of commercially available yeast paste of *Saccharomyces cerevisiae* (containing 10 g of dry yeast) were resuspended in 170 g of a hypertonic solution of NaCl 2.0M (116.88 g/l of sodium chloride and 29.41 g/l of trisodium citrate·$2H_2O$) and kept at 25°C for 6 hours under gentle stirring.

Step II) - The cells were already inactivated by the hypertonic treatment of Step I), and consequently a further inactivation by means of chemico-physical systems was not required.

Step III) - From the suspension obtained in Step I), an aliquot of 80 g was taken, and this was centrifuged at 4000 r.p.m. (1252 R.C.F.) for 15 minutes. The centrifugate obtained was washed with 80 ml of an isotonic solution of ascorbic acid (59.4 g/l), and the cells were recovered and brought back up to 80 g with an isotonic solution of ascorbic acid.

The suspension was kept for 6 hours at 25°C under gentle stirring.

The cells obtained may be stored up to the moment of use or may be analytically tested for the quantity of ascorbic acid incorporated.

*Analytical test of the suspensions obtained*

The suspensions obtained as described above were tested in order to check the

quantity of ascorbic acid that had accumulated in the cells.

Step IV) - The suspension obtained in Step III) was centrifuged at 4000 r.p.m. (1252 R.C.F.). The precipitated was washed twice (sample A) or thrice (sample B) with an isotonic solution of sodium chloride (0.9%) keeping it in suspension during each washing.

Step V) - The centrifugate was brought up to 80 g with the hypertonic solution (NaCl 2.0M) used for Step I). In order to complete the osmotic treatment, the suspension was kept under gentle stirring for 6 hours at 25°C.

Step VI) – The ascorbic acid present in each solution (mg/ml) and in the microorganism (mg/g) was determined by HPLC, by the use of the following formula:

$mg/g = A_c \times P_s \times f_s \times D_s \times 1000 / A_s \times P_c \times D_c$,

where:

$A_c$ = Sample Absorbance

$P_s$ = Standard weight

$f_s$ = Standard factor

$D_s$ = Standard dilution

$A_s$ = Standard absorbance

$P_c$ = Sample weight

$D_c$ = Sample dilution

Step VII) - *Determination of cell viability*

A small part (1 g) of the centrifugate obtained in Step IV) was then used to ascertain the viability of the cells, using the microbiological technique of fermentation of saccharose in a 9.25% isotonic solution and counting of the viable microorganisms on Platecount agar. The Total Viable Cells (TVC) method was used, which is commonly known and described in microbiology manuals, such as Berkley's Manual, and Trattato di Microbiologia, Davis and Dulbecco, Piccin Ed., Padova. The results presented in table corresponding to *Step VI* show that no viable cells could be detected after 24 hours of growth, indicating that the microorganisms have been inactivated by the previous treatment in hypertonic solution.

Test 2NC-A,B

*Step I* **Treatment with hypertonic solution**

| Test | S. cerevisiae Weight [g] * | Hypertonic solution | NaCl concentr. [%] | time [hours] | temperature [°C] |
|---|---|---|---|---|---|
| **2NC-A** | 15.00 | 185.00 | 11.69 | 6 | 25 |
| **2NC-B** | 15.00 | 185.00 | 11.69 | 6 | 25 |

* Weighing carried out on substance as such, loss due to drying 63.40%

*Step II* **Treatment with isotonic solution of ascorbic acid**

| Test | Centrifugate Weight [g] | Washed w.isotonic soln [g] | centrifugate Weight [g] | isot. Soln. added Weight [g] | Time [hours] | Temperature [°C] |
|---|---|---|---|---|---|---|
| **2NC-A** | 9.51 | 70.49 | 13.05 | 66.95 | 6 | 25 |
| **2NC-B** | 9.63 | 70.37 | 13.01 | 66.99 | 6 | 25 |

*Step III* **Washing with NaCl (0.9%)isotonic solution**

| Test | centrifugate Weight [g] | washed 1X with isot. soln Weight [g] | centrifugate Weight [g] | washed 2X with isot. soln Weight [g] | centrifugate Weight [g] | washed 3X isot. soln Weight [g] |
|---|---|---|---|---|---|---|
| **2 NC-A** | 10.67 | 69.33 | 11.06 | 68.94 | 10.93 | |
| **2NC-B** | 10.79 | 69.21 | 11.15 | 68.85 | 11.10 | 68.90 |

*Step IV* **Treatment with hypertonic solution to release the incorporated ascorbate**

| Test | centrifugate Weight [g] | Hypertonic soln added | centrifugate Weight [g] | time [hours] | temperature [°C] |
|---|---|---|---|---|---|
| **2NC-A** | 10.93 | 69.07 | 9.05 | 6 | 25 |
| **2NC-B** | 10.95 | 69.05 | 8.97 | 6 | 25 |

*Step V* **Incorporated ascorbic acid as measured by HPLC**

**HPLC data**

| Batch | 2NC-A | 2NC-B |
|---|---|---|
| Ac | 3373.79 | 2964.39 |
| Ps | 59.4 | 59.4 |
| Fs | 1 | 1 |
| Ds | 0.0005 | 0.0005 |
| As | 2488.52 | 2488.52 |
| Pc | 4000 | 4000 |
| Dc | 0.011842 | 0.011842 |

$$‰\ (mg/g) = \frac{Ac \times Ps \times fs \times Ds \times 1000}{As \times Pc \times Dc}$$

**Incorporated Ascorbic acid**

| Ascorbic acid | A | B | |
|---|---|---|---|
| mg/ g * | **0.850** | **0.747** | |

* The amount of ascorbic acid released by 1g of yeast over dry matter by osmotic treatment is highlighted in bold type.

*Step VI* **Results of biological activity**

| Test | fermentat. of saccharose* after 24 h | |
|---|---|---|
| **A,B** | negative | |

* Saccharose concentration 9.25% (isotonic)

**Example 2: Yeasts modified by incorporation of oxytetracycline**

The same experiment performed in example 1, was repeated loading yeasts cells

with oxytetracycline.

Step I) – 30 g of commercially available yeast paste of *Saccharomyces cerevisiae* (containing 10 g of dry yeast) were suspended in 170 g of a hypertonic solution of NaCl 2.0M (116.88 g/l of sodium chloride and 29.41 g/l of sodium citrate $2H_2O$) and kept at 25°C for 6 hours under gentle stirring.

Step II) - The cells were already inactivated by the hypertonic treatment of Step I), and consequently a further inactivation by means of chemico-physical systems was not required.

Step III) - From the suspension obtained in Step I), an aliquot of 80 g was taken, this was centrifuged at 4000 r.p.m. (1252 R.C.F.) for 15 minutes. The centrifugate obtained was washed with 80 ml of an isotonic solution of oxytetracycline HCl (32.6 g/l) and citric acid (27.6 g/l), and the cells were recovered and brought back up to 80 g with an isotonic solution as above. The suspensions were kept for 6 hours at 25°C under gentle stirring.

The cells obtained may be stored up to the moment of use or may be analytically tested for the quantity of oxytetracycline loaded.

*Analytical test of the suspensions obtained*

The suspension obtained as described above was tested in order to check the quantity of oxytetracycline that had accumulated in the cells.

Step IV) - The suspension obtained in Step III) was centrifuged at 4000 r.p.m. (1252 R.C.F.). The centrifugate was washed twice (A) or thrice (B) with an isotonic solution of sodium chloride (0.9%) keeping it in suspension for 10 minutes before centrifugation.

Step V) - The centrifugate was brought up to 80 g with the hypertonic solution (NaCl 2.0M) used for Step I). In order to complete the osmotic treatment, the suspension was kept under gentle stirring for 6 hours at 25°C.

Step VI) – The oxytetracycline HCl present in each solution (mg/ml) and in the microorganism (mg/g) was determined by HPLC, by the use of the following formula:

$mg/g = A_c \times P_s \times f_s \times D_s \times 1000 / A_s \times P_c \times D_c$,

where:

$A_c$ = Sample Absorbance

$P_s$ = Standard weight

$f_s$ = Standard factor

$D_s$ = Standard dilution

$A_s$ = Standard absorbance

$P_c$ = Sample weight

$D_c$ = Sample dilution

Step VII) - *Determination of cell viability*

A small part (1 g) of the centrifugate obtained in Step IV) was then used to ascertain the vitality of the cells, using the microbiological technique of fermentation of saccharose in a 9.25% isotonic solution and counting of the vital germs with Platecount agar. The results presented in table corresponding to *Step VII* show that no viable cells could be detected after 24 hours of growth, indicating that the microorganisms have been inactivated by the previous treatment in hypertonic solution.

Test 3NO-A,B

*Step I* **Treatment with hypertonic solution**

| Test | *S. cerevisiae* Weight [g]* | hypertonic solution Weight [g] | NaCl concentr. [%] | time [hours] | temperature [°C] |
|---|---|---|---|---|---|
| A,B | 30.00 | 170.00 | 11.69 | 6 | 25 |

* Loss due to drying 63.40%

*Step III* **Treatment with isotonic solution containing oxytetracycline HCl and citric acid**

| Test | Centrifugate Weight [g] | washed with oxytetracycl. Weight [g] * | Centrifugate Weight [g] | oxytetracycl. added Weight [g] | time [hours] | Temp. [°C] |
|---|---|---|---|---|---|---|
| **3NO-A** | 9.34 | 70.66 | 13.07 | 66.93 | 6 | 25 |
| **3NO-B** | 9.27 | 70.73 | 13.22 | 66.76 | 6 | 25 |

* isotonic solution containing 3.26% of oxytetracycline HCl and 2.76% of citric acid

*Step IV* **Washing with NaCl (0.9%) isotonic solution**

| Test | centrifugate Weight [g] | 1X washing. NaCl soln Weight [g] | Centrifugate Weight [g] | 2X washings NaCl soln Weight [g] | centrifugate Weight [g] | 3X washings NaCl soln Weight [g] |
|---|---|---|---|---|---|---|
| **3NO-A** | 14.40 | 65.60 | 13.79 | 66.21 | 13.10 | |
| **3NO-B** | 14.54 | 65.46 | 14.10 | 65.90 | 13.45 | 66.55 |

Step V **Hypertonic solution treatment**

| Test | centrifugate Weight [g] | hypertonic solution ad Weight [g] | centrifugate Weight [g] | time [hours] | Temp. [°C] |
|---|---|---|---|---|---|
| **3NO-A** | 13.10 | 66.99 | 9.05 | 6 | 25 |
| **3NO-B** | 13.55 | 66.45 | 8.97 | 6 | 25 |

*Step VI* **Incorporated Oxytetracycline as measured by HPLC**

**HPLC data**

| Batch | 3NO-A | 3NO-B |
|---|---|---|
| Ac | 3309.00 | 3180.09 |
| Ps | 32.6 | 32.6 |
| Fs | 1 | 1 |
| Ds | 0.001 | 0.001 |
| As | 480.63 | 480.63 |
| Pc | 4000 | 4000 |
| Dc | 0.118 | 0.118 |

$$‰ (mg/g) = \frac{Ac \times Ps \times fs \times Ds \times 1000}{As \times Pc \times Dc}$$

**Incorporated oxytetracyclin**

| | Oxytetracyclin | final soln. | | |
|---|---|---|---|---|
| washings | mg/ ml | 0.249 | 2.363 | 0.920 |
| A | mg/ g *) | **4.738** | | |
| B | mg/ g *) | **4.554** | | |

*) The amount of oxytetracycline released by 1g of yeast over dry matter by osmotic treatment is highlighted in bold type.

*Step VII* **Results of biological activity**

| Test | fermentat. of saccharose* after 24 h |
|---|---|
| A,B | negative |

* Saccharose concentration 9.25% (isotonic)

**Example 3: Yeasts modified by incorporation of sodium sulphadimethoxine**

Yeast cells have been emptied by hypertonic solution treatment and then loaded

with an isotonic sodium sulphadimethoxine solution.

Step I) – 30 g of commercially available yeast paste of *Saccharomyces cerevisiae* (containing 10 g of dry yeast) were suspended in 170 g of a hypertonic solution of NaCl 2.0M (116.88 g/l of sodium chloride and 29.41 g/l of sodium citrate $2H_2O$) and kept at 25°C for 6 hours under gentle stirring.

Step II) - The cells were already inactivated by the hypertonic treatment of Step I), and consequently a further inactivation by means of chemico-physical systems was not required.

Step III) - From the suspension obtained in Step I), an aliquot of 80 g was taken, and this was centrifuged at 4000 r.p.m. (1252 R.C.F.) for 15 minutes. The centrifugate obtained was washed with 80 ml of an isotonic solution of sodium sulphadimethoxine (51.7 g/l), and the cells were recovered and brought back up to 80 g with an isotonic solution of sodium sulphadimethoxine and sodium citrate 0.025M. The suspension was kept for 6 hours at 25°C under gentle stirring.

The cells obtained may be stored up to the moment of use or may be tested for the quantity of sulphadimethoxine that has been loaded.

*Analytical test of the suspensions obtained*

The suspension obtained as described above was tested in order to check the quantity of sodium sulphadimethoxine that had accumulated in the cells.

Step IV) - The suspension obtained in Step III) was centrifuged at 4000 r.p.m. (1252 R.C.F.). The centrifugate was washed twice (A) or thrice (B) with an isotonic solution of sodium chloride (0.9%) keeping it in suspension for 10 minutes before centrifugation.

Step V) - The centrifugate was brought up to 80 g with the hypertonic solution (NaCl 2.0M) used for Step I). In order to complete the osmotic treatment, the suspension was kept under gentle stirring for 6 hours at 25°C.

Step VI) – The sodium sulphadimethoxine present in each (mg/ml) and in the microorganism (mg/g) was determined by HPLC, by the use of the following formula:

$mg/g = A_c \times P_s \times f_s \times D_s \times 1000 / A_s \times P_c \times D_c$,

where:

$A_c$ = Sample Absorbance

$P_s$ = Standard weight

$f_s$ = Standard factor

$D_s$ = Standard dilution

$A_s$ = Standard absorbance

$P_c$ = Sample weight

$D_c$ = Sample dilution

Step VII) - *Determination of cell viability.*

A small part (1 g) of the centrifugate obtained in Step IV) was then used to ascertain the vitality of the cells, using the microbiological technique of fermentation of saccharose in a 9.25% isotonic solution and counting of the vital microorganisms (C.F.U.) with Platecount agar. The results presented in table corresponding to *Step VII* show that no viable cells could be detected after 24 hours of growth, indicating that the microorganisms have been inactivated by the previous treatment in hypertonic solution.

**Test 4NS1-A,B**

*Step I* **Treatment with hypertonic solution**

| Test | yeast *S. cerevisiae* Weight [g]* | hypertonic solution Weight [g] | NaCl concentr. [%] | time [hours] | temperature [°C] |
|---|---|---|---|---|---|
| A,B | 30.00 | 170.00 | 11.69 | 6 | 25 |

* Weighing carried out on substance as such, loss due to drying: 63.40%

*Step III* **Treatment with sodium sulphadimethoxine isotonic solution**

| Test | centrifugate Weight [g] | Isotonic soln. wash Weight [g] | centrifugate Weight [g] | Isotonic soln. added Weight [g] | time [hours] | temperature [°C] |
|---|---|---|---|---|---|---|
| **4NS1-A** | 9.62 | 70.38 | 12.42 | 67.58 | 6 | 25 |
| **4NS1-B** | 9.64 | 70.36 | 12.50 | 67.50 | 6 | 25 |

*Step IV* **Washing with NaCl (0.9%) isotonic solution**

| Test | centrifugate Weight [g] | 1X washing Weight [g] | centrifugate Weight [g] | 2X washing Weight [g] | centrifugate Weight [g] | 3X washing Weight [g] |
|---|---|---|---|---|---|---|
| **4NS1-A** | 13.57 | 66.43 | 13.32 | 66.68 | 13.25 | |
| **4NS1-B** | 13.41 | 66.59 | 13.27 | 66.73 | 13.19 | 66.81 |

*Step V* **Treatment with hypertonic solution to release loaded sulphadimethoxine**

| Test | centrifugate Weight [g] | hypertonic solution Weight [g] | centrifugate Weight [g] | time [hours] | Temperature [°C] |
|---|---|---|---|---|---|
| **4NS1-A** | 13.25 | 66.75 | 9.92 | 6 | 25 |
| **4NS1-B** | 12.69 | 67.31 | 10.06 | 6 | 25 |

*Step VI* **Incorporated sulphadimetoxin as measured by HPLC**

**HPLC data**

| Batch | 4NS1-A | 4NS1-B |
|---|---|---|
| Ac | 2266.98 | 1830.04 |
| Ps | 51.7 | 51.7 |
| Fs | 1 | 1 |
| Ds | 0.001 | 0.001 |
| As | 1741.13 | 1741.13 |
| Pc | 4000 | 4000 |
| Dc | 0.0118 | 0.0118 |

$$‰ (mg/g) = \frac{Ac \times Ps \times fs \times Ds \times 1000}{As \times Pc \times Dc}$$

**Incorporated sulphadimetoxin**

| washings | mg/ml | 0.075 | 4.440 | 0.424 |
|---|---|---|---|---|
| A | mg/ g* | **1.421** | | |
| B | mg/ g * | **1.147** | | |

* The amount of sulphadimethoxine released by 1g of yeast over dry matter by osmotic treatment is highlighted in bold type.

*Step VII* **Results of biological activity**

| Test | fermentat. of saccharose* after 24 h |
|---|---|
| A,B | negative |

* Saccharose concentration 9.25% (isotonic)

**Example 4: Yeasts modified by incorporation of sodium sulphadimethoxin (hypertonicity due to the drug).**

In this example is shown that is possible to induce the hypertonic shock by an hypertonic solution of the sodium salt of the drug itself.

Step I) – To 30 g of commercially available yeast paste of *Saccharomyces cerevisiae* (containing 20 g of water) were added 3.32 g of sodium sulphadimethoxin, 0.29 g of sodium citrate · $2H_2O$ and 8.5 g of water under gentle stirring. The suspension obtained was kept at 25°C for 6 hours.

Step II) - The cells were already inactivated by the hypertonic solution treatment, and consequently further inactivation by means of chemico-physical systems was not required.

Step III) - The suspension obtained in Step I) was diluted with 30.61 g of distilled water at isotonicity, and the suspension was kept under gentle stirring for 6 hours at 25°C.

The cells obtained may be stored up to the moment use or tested for the quantity of sulphadimethoxin that has penetrated into them.

*Analytical test of the suspension obtained*

The suspension as previously obtained was tested to ascertain the quantity of sodium sulphadimethoxin that had accumulated in the cells.

Step IV) - For analytical reasons, the suspension obtained in Step III) was diluted with an isotonic solution of sodium sulphadimethoxin (5.17%) to 200 g. An aliquot of 80 g of suspension was taken. The suspension was centrifuged at 10 000 r.p.m., and the centrifugate was washed twice (A) or thrice (B) with an isotonic solution of sodium chloride (0.9%). The suspension was stirred for 10 minutes before each washing.

Step V) - The centrifugate was brought to 80 g with a hypertonic solution of NaCl 2.0M (116.88 g/l of sodium chloride and 29.41 g/l of sodium citrate $2H_2O$) under gentle stirring for 6 hours at 25°C.

Step VI) - The sodium sulphadimethoxin present in solution (mg/ml) and in the microorganism (mg/g) was determined by HPLC, by the use of the following formula:

$mg/g = A_c \times P_s \times f_s \times D_s \times 1000 / A_s \times P_c \times D_c$,

where:

$A_c$ = Sample Absorbance

$P_s$ = Standard weight

$f_s$ = Standard factor

$D_s$ = Standard dilution

$A_s$ = Standard absorbance

$P_c$ = Sample weight

$D_c$ = Sample dilution

The values obtained were similar to those obtained in the previous example.

Step VII) - *Determination of cell viability*

A small part (1 g) of the centrifugate obtained in Step IV) was then used to ascertain viability of the cells, using the microbiological technique of fermentation of saccharose in a 9.25% isotonic solution and counting of the vital germs with Platecount agar. The results presented in table corresponding to *Step VII* show that no viable cells could be detected after 24 hours of growth, indicating that the microorganisms have been inactivated by the previous treatment in hypertonic solution.

**Test 6NS1-A,B**

*Step I* **Treatment with hypertonic solution of sodium sulphadimethoxin**

| Test | strain *S. cerevisiae* Weight [g]* | sulphadim. added Weight [g] | Na-citrate added Weight [g] | water added Weight [g] | sulphadim. concentration [%] | time [hours] | temperature [°C] |
|---|---|---|---|---|---|---|---|
| A,B | 30.00 | 3.32 | 0.29 | 8.50 | 10.34 | 6 | 25 |

* Weighing carried out on the substance as such (containing 10 g of dry yeast and 20 g of water)

*Step III* **Treatment with isotonic solution of sodium sulphadimethoxin**

| Test | water added Weight [g] | sulphadim. concentr. [%] | Time [hours] | Temperature [°C] |
|---|---|---|---|---|
| A,B | 30.61 | 5.17 | 6 | 25 |

*Step IV* **Washing with NaCl (0.9%) isotonic solution**

| Test | centrifugate Weight [g] | 1X washing Weight [g] | centrifugate Weight [g] | 2X washing Weight [g] | centrifugate Weight [g] | 3X washing Weight [g] |
|---|---|---|---|---|---|---|
| **6NS1-A** | 16.25 | 63.75 | 15.81 | 64.19 | 15.99 | |
| **6NS1-B** | 16.20 | 63.80 | 15.21 | 64.79 | 15.41 | 64.59 |

*Step V* **Treatment with hypertonic solution(*) to release the sulphadimethoxin incorporated in the cells**

| Test | centrifugate Weight [g] | hypertonic solution* Weight [g] | time [hours] | Temperature [°C] | centrifugate Weight [g] |
|---|---|---|---|---|---|
| **6NS1-A** | 15.99 | 64.01 | 6 | 25 | 11.81 |
| **6NS1-B** | 15.47 | 64.53 | 6 | 25 | 11.22 |

* hypertonic solution (116.88 g/l of sodium chloride and 29.41 g/l of sodium citrate $2H_2O$)

*Step VI* **Incorporated sodium sulphadimetoxin as measured by HPLC**

**HPLC data**

| Batch | 6NS1-A | 6NS1-B |
|---|---|---|
| Ac | 2049.23 | 1908.71 |
| Ps | 51.7 | 51.7 |
| Fs | 1 | 1 |
| Ds | 0.001 | 0.001 |
| As | 1782.36 | 1782.36 |
| Pc | 4000 | 4000 |
| Dc | 0.0118 | 0.0118 |

$$‰\ (mg/g) = \frac{Ac \times Ps \times fs \times Ds \times 1000}{As \times Pc \times Dc}$$

Sulphadimetoxin final soln.

| | | | | |
|---|---|---|---|---|
| washings | mg / ml | 0.066 | 3.741 | 0.275 |
| A | mg / g * | **1.255** | | |
| B | mg / g * | **1.169** | | |

* The quantity of sulphadimethoxin released by 1g of yeast over dry matter by osmotic treatment is highlighted in bold type.

*Step VII* **Residual biological activity**

| Test | fermentat. of saccharose* after 24 h |
|---|---|
| A,B | negative |

* Saccharose concentration 9.25% (isotonic)

**Example 5: Yeasts modified by incorporation of ascorbic acid followed by thermal stabilization.**

The incorporation was repeated as in Example 1, with in addition thermal stabilization of the modified yeast.

Step I) – 30 g of commercially available yeast paste of *Saccharomyces cerevisiae* (corresponding to 10 g of dried yeast) was suspended in 170 g of a hypertonic solution of NaCl 2.0M (116.88 g/l of sodium chloride and 29.41 g/l of sodium citrate $2H_2O$) and kept at 25°C for 6 hours under gentle stirring.

Step II) - The cells were already inactivated by the hypertonic treatment of Step I), and consequently a further inactivation by means of chemico-physical systems was not required.

Step III) - From the suspension obtained in Step I), an aliquot of 80 g was taken, and this was centrifuged at 4000 r.p.m. (1252 R.C.F.) for 15 minutes. The centrifugate obtained was washed with 80 ml of an ascorbic acid isotonic solution (59.4 g/l), and cells were recovered and brought back up to 80 g with the same isotonic solution.

The suspension was kept for 6 hours at 25°C under gentle stirring.

The cells obtained may be stored up to the moment of use or may be tested for the quantity of ascorbic acid that has penetrated into them.

*Analytical test of the suspension obtained*

The suspension obtained as described above was tested in order to check the quantity of ascorbic acid that had been loaded in the cells.

Step IV) - The suspension obtained in Step III) was centrifuged at 4 000 r.p.m. (1252 R.C.F.). The centrifugate was washed twice with an isotonic solution of sodium chloride (0.9%) keeping it in suspension for 10 minutes before centrifugation. The centrifugate was vacuum-dried on silica gel at room temperature (A).

Step V) - The dried yeast was either used as such (A) or an aliquot (1 g) heated for 15 minutes at 120°C (B). Both fraction were treated with 10 ml of a hypertonic solution as in Step I). To complete the osmotic treatment, the suspension was kept under gentle stirring for 6 hours at 25°C.

Step VI) – The ascorbic acid present in the washing solution (mg/ml) and in the

microorganism (mg/g) was determined by HPLC, by the use of the following formula:

$mg/g = A_c \times P_s \times f_s \times D_s \times 1000 / A_s \times P_c \times D_c$,

where: $A_c$ = Sample Absorbance

$P_s$ = Standard weight

$f_s$ = Standard factor

$D_s$ = Standard dilution

$A_s$ = Standard absorbance

$P_c$ = Sample weight

$D_c$ = Sample dilution

Step VII) - *Determination of cell viability*

A small part (1 g) of the centrifugates obtained in Step IV) was used to ascertain the vitality of the cells, using the microbiological technique of fermentation of saccharose in a 9.25% isotonic solution and counting of the viable microorganisms with Platecount agar. The results presented in table corresponding to *Step VII* show that no viable cells could be detected after 24 hours of growth, indicating that the microorganisms have been inactivated by the previous treatment in hypertonic solution.

**Test 5NC-A,B**

*Step I* **Treatment with hypertonic solution**

| Test | S. cerevisiae Weight [g]* | hypertonic solution Weight [g] | NaCl concentr. [%] | time [hours] | temperature [°C] |
|---|---|---|---|---|---|
| A,B | 15.00 | 185.00 | 11.69 | 6 | 25 |

* Weighing carried out on substance as such

*Step III* **Treatment with isotonic solution of ascorbic acid**

| Test | Centrifugate Weight [g] | washing with isotonic soln. Weight [g] | Centrifugate Weight [g] | Isotonic soln. added Weight [g] | time [hours] | Temperature [°C] |
|---|---|---|---|---|---|---|
| **5NC** | 9.52 | 70.48 | 12.81 | 67.19 | 6 | 25 |

*Step IV* **Washing with NaCl (0.9%)isotonic solution and drying**

| Test | centrifugate Weight [g] | 1X wash NaCl 0.9% Weight [g] | centrifugate Weight [g] | 2X wash NaCl 0.9% Weight [g] | centrifugate Weight [g] | Dry yeast Weight [g] * |
|---|---|---|---|---|---|---|
| **5NC** | 10.66 | 69.14 | 10.77 | 69.23 | 10.85 | 2.81 |

* Loss due to transfer into drying capsule

*Step V* **Treatment with hypertonic solution to release incorporated ascorbate**

| Test | Dry yeast Weight [g] | Hypertonic Soln. Weight [g] | time [hours] | Temperature [°C] |
|---|---|---|---|---|
| **A** | 1.17 | 11.17 | 6 | 25 |
| **B** | 1.00 | 11.00 | | |

*Step VI* **Incorporated ascorbic acid as measured by HPLC**

**HPLC data**

| Batch | 5NC-A | 5NC-B |
|---|---|---|
| Ac | 7760.00 | 6305.3 |
| Ps | 59.4 | 59.4 |
| Fs | 1 | 1 |
| Ds | 0.0005 | 0.0005 |
| As | 3182.27 | 3182.27 |
| Pc | 1170 | 1000 |
| Dc | 0.045 | 0.045 |

$$‰\ (mg/g) = \frac{Ac \times Ps \times fs \times Ds \times 1000}{As \times Pc \times Dc}$$

**Incorporated ascorbic acid**

| | Ascorbic acid | final soln. | | |
|---|---|---|---|---|
| washings | mg/ ml | 0.16 | 1.452 | 0.147 |
| A | mg/ g * | **1.376** | | |
| B | mg/ g * | **1.308** | | |

* The quantity of ascorbic acid released by 1 g of yeast over dry matter by osmotic treatment is highlighted in bold type.

*Step VII* **Results of biological activity**

| Test | fermentat. of saccharose* after 24 h |
|---|---|
| A,B | negative |

* Saccharose concentration 9.25% (isotonic)

**Example 6: Yeasts modified by incorporation of ascorbic acid in isotonic solution**

Step I) 30 g of yeast paste as in Example 5 were dissolved in an aqueous solution,

and the yeast was inactivated by thermal treatment, at 65°C for 30 minutes.

Step II) The inactivated cells were re-suspended in NaCl 0.9% isotonic solution (80 ml) comprising the ascorbic acid (59.4 g/l) to be incorporated.

Step III) The suspension was left under stirring for 60 hours in the same conditions as for the previous examples.

Step IV) Finally, centrifugation was carried out, as in the previous examples.

Step V) The product was buffered with a 1% glutaraldehyde solution, as stabilizer for the cells, and thus to stop and limit the leakage of the ascorbic acid from the cells.

**Test 6NC**

**Ascorbic acid quantity as measured by HPLC**

Ascorbic acid final soln. washings

| | | | | |
|---|---|---|---|---|
| mg/ ml | 0.038 | 3.124 | | |
| mg/ g * | **0.717** | | | |

* The quantity of ascorbic acid released by 1g of yeast over dry matter by osmotic treatment is highlighted in bold type.

**Results of biological activity**

| Test | fermentat. of saccharose* after 24 h |
|---|---|
| | negative |

**Example 7: Microorganism modified by incorporation of zinc.**

In this treatment yeast cells have been partially emptied by the hypertonic solution, comprising NaCl and Trisodium citrate bihydrate, as already described. Zinc was then incorporated by incubation in a $ZnSO_4$ isotonic solution. The quantity of zinc loaded into the cells, was measured by Atomic Absorbance Spectrometry (AAS).

Step I) – 1680 g of commercially available yeast paste of *Saccharomyces cerevisiae* (corresponding to 560 g of dried yeast) was suspended in 5 600 g of a hypertonic solution of NaCl 1.0M (58.44 g/l of sodium chloride and 9.8 g/l of sodium citrate $2H_2O$) and kept at 25°C for 16 hours under gentle stirring.

Step II) - The cells were already inactivated by the hypertonic treatment of Step I), and consequently a further inactivation by means of chemico-physical systems was not required.

Step III) – The cellular suspension was centrifuged at 4500 r.p.m. (4596 RCF) for 15 minutes. The centrifugate obtained was washed once with 5.6 L zinc hypotonic solution (80% isotonic) and then with 5.6 L isotonic Zn solution ($ZnSO_4 \cdot H2O$ 45.2 g/l).

The suspension was kept for 6 hours at 25°C under gentle stirring.

*Analytical test of the suspension obtained*

The suspension obtained as described above was fixed with formaldheyde as follows: 28g of 37% formaldheyde were added to the suspension and stirred for 2 hours at 20-25°C. The supension was then centrifuged and the centrifugate was washed twice with 5.6 L NaCl isotonic solution (0.9%).

Step IV) - The centrifugate was air-dried at 45°C for 36 hours. After mincing the dried pellets were sifted on a 400μ net.

Step V) – The incorporated zinc quantity was determined by AAS after 5N HCl extraction for two hours at 25°C under gentle stirring.

Step VI) - Determination of cell viability

A small part (1 g) of the centrifugates obtained in Step IV) was used to ascertain the vitality of the cells, using the microbiological technique of fermentation of saccharose in a 9.25% isotonic solution and counting of the viable microorganisms with Platecount agar. The results presented in table corresponding to *Step VI* show that no viable cells could be detected after 24 hours of growth, indicating that the microorganisms have been inactivated by the previous treatment in hypertonic solution

Step V: Data from AA Spectrum

**Test 7/5600**

**AAS data**

| | |
|---|---|
| Batch | 7/5600 |
| Ac | 0.209 |
| Ps | 66.0 |
| Fs | 0.805 |
| Ds | 0.00006 |
| As | 0.239 |
| Pc | 2500 |
| Dc | 0.0008 |

Incorporated zinc

| | |
|---|---|
| Measurement unit | ‰ |
| Content | **1.39** |
| Yield (g) | 514 |
| Washings No. | 2 |

$$‰\ (mg/g) = \frac{Ac \times Ps \times fs \times Ds \times 1000}{As \times Pc \times Dc}$$

Ac: Sample absorbance

Ps: Standard weight

Fs: Standard factor

Ds: Standard dilution

As: Standard absorbance

Pc: Sample weight

Dc: Sample weight

*Step VI* **Results of biological activity**

| Test | fermentat. of saccharose* after 24 h |
|---|---|
| | negative |

* Saccharose concentration 9.25% (isotonic)

**Example 8: Microorganism modified by incorporation of zinc and cobalt.**

In this treatment yeast cells have been partially emptied by the hypertonic solution, comprising NaCl and Trisodium citrate bihydrate, as already described. Zinc and cobalt were then incorporated by incubation in a $ZnSO_4$ and $CoSO_4$ hypotonic solution. The quantity of zinc and cobalt loaded into the cells, was measured by Atomic Absorbance Spectrometry (AAS).

Step I) – 168 g of commercially available yeast paste of *Saccharomyces cerevisiae* (corresponding to 56 g of dried yeast) was suspended in 560 g of a hypertonic solution of NaCl 2.0M (58.45 g/l of sodium chloride and 9.80 g/l of trisodium citrate $2H_2O$) and kept at 25°C for 16 hours under gentle stirring.

Step II) - The cells were already inactivated by the hypertonic treatment of Step I), and consequently a further inactivation by means of chemico-physical systems was not required.

Step III) – The cellular suspension was centrifuged at 4500 r.p.m. (4596 RCF) for 15 minutes. The centrifugate obtained was washed twice with 560 g zinc/cobalt hypotonic solution (prepared as follows: $ZnSO_4$ 32.54 g/l, $CoSO_4$ 5.69 g/l). The suspension was resuspended in 560 g of the same solution and kept 16 hours at 25°C under gentle stirring.

*Analytical test of the suspension obtained*

The suspension was divided in two aliquots and then either fixed with formaldheyde (37B) or not (37A) as follows: 1.4 g 37% formaldheyde were added (or not) to the suspension and stirred for 2 hours at 20-25°C. The suspensions were then centrifuged and the centrifugates were washed twice with 280 g NaCl isotonic solution (0.9%).

Step IV) - The centrifugate was air-dried at 45°C for 36 hours. After mincing the dried pellets were sifted on a 400μ net.

Step V) – The incorporated zinc quantity was determined by AAS after 5N HCl extraction for two hours at 25°C under gentle stirring.

Step VI) - Determination of cell viability

A small part (1 g) of the centrifugates obtained in Step IV) was used to ascertain the vitality of the cells, using the microbiological technique of fermentation of saccharose in a 9.25% isotonic solution and counting of the viable microorganisms with Platecount agar. The results presented in table corresponding to *Step VI* show that no viable cells could be detected after 24 hours of growth, indicating that the microorganisms have been inactivated by the previous treatment in hypertonic solution

Step V: Incorporated zinc and cobalt as measured by AA Spectrum

**Test 37A and 37B**

**AAS data**

| | Zn | Co | Zn | Co |
|---|---|---|---|---|
| Batch | 37A/560 | 37A/560 | 37B/560 | 37B/560 |
| Ac | 0.114 | 0.031 | 0.115 | 0.031 |
| Ps | 66.0 | 49.0 | 66.0 | 49.0 |
| Fs | 0.805 | 0.2085 | 0.805 | 0.2085 |
| Ds | 0.00006 | 0.00025 | 0.00006 | 0.0025 |
| As | 0.193 | 0.049 | 0.193 | 0.049 |
| Pc | 2500 | 2500 | 2500 | 2500 |
| Dc | 0.0008 | 0.008 | 0.0008 | 0.008 |

| Measurement unit | ‰ | ‰ | ‰ | ‰ |
|---|---|---|---|---|
| Content | 0.94 | 0.08 | 0.95 | 0.08 |
| Zn/Co | 11.65 | 1 | 11.76 | 1 |
| Yield (g) | 20.77 | 20.77 | 19.25 | 19.25 |
| Washings No. | 2 | 2 | 2 | 2 |

$$‰ (mg/g) = \frac{Ac \times Ps \times fs \times Ds \times 1000}{As \times Pc \times Dc}$$

Ac: Sample absorbance
Ps: Standard weight
Fs: Standard factor
Ds: Standard dilution
As: Standard absorbance
Pc: Sample weight
Dc: Sample weight

**Example 9: Microorganism modified by incorporation of zinc and cobalt.**

In this treatment yeast cells have been partially emptied by the hypertonic solution, comprising NaCl and Trisodium citrate bihydrate, as already described. Zinc and cobalt were then incorporated by incubation in a $ZnSO_4$ and $CoSO_4$ hypotonic solution. The quantity of zinc and cobalt loaded into the cells, was measured by Atomic Absorbance Spectrometry (AAS).

Step I) – 168 g of commercially available yeast paste of *Saccharomyces cerevisiae* (corresponding to 56 g of dried yeast) was suspended in 560 g of a hypertonic solution containing 29.46 g NaCl and 4.94 g trisodium citrate·$2H_2O$) and kept at 25°C for 16 hours under gentle stirring.

Step II) - The cells were already inactivated by the hypertonic treatment of Step I), and consequently a further inactivation by means of chemico-physical systems was not required.

Step III) – The cellular suspension was centrifuged at 4500 r.p.m. (4596 RCF) for 15 minutes. The centrifugate obtained was washed twice with 560 g zinc/cobalt hypotonic solution containing: 32.54 g $ZnSO_4$, 5.69 g $CoSO_4$ ·$7H_2O$. The suspension was resuspended in 560 g of the same solution and kept 16 hours at 25°C under gentle stirring.

*Analytical test of the suspension obtained*

The suspension was then centrifuged and the centrifugates were washed twice with 560 g NaCl isotonic solution (0.9%).

Step IV) - The centrifugate was air-dried at 45°C for 36 hours. After mincing the

dried mass of cells was sifted on a 400μ net.

Step V) – The incorporated zinc and cobalt quantities were determined by AAS after 5N HCl extraction for two hours at 25°C under gentle stirring. In this case (test 38) the quantity of Zinc and Cobalt are higher than in the previous test (37A and B) due to a more effective fixing treatment in test 38.

Step VI) - Determination of cell viability

A small part (1 g) of the centrifugates obtained in Step IV) was used to ascertain the vitality of the cells, using the microbiological technique of fermentation of saccharose in a 9.25% isotonic solution and counting of the viable microorganisms with Platecount agar. The results presented in table corresponding to *Step VI* show that no viable cells could be detected after 24 hours of growth, indicating that the microorganisms have been inactivated by the previous treatment in hypertonic solution

Step V: Incorporated zinc and cobalt as measured by AASpectrum

**Test 38/560**

**AAS data**

| | Zn | Co |
|---|---|---|
| Batch | 38/560 | 38/560 |
| Ac | 0.142 | 0.037 |
| Ps | 66.0 | 49.0 |
| Fs | 0.805 | 0.2085 |
| Ds | 0.00006 | 0.00025 |
| As | 0.193 | 0.049 |
| Pc | 2500 | 2500 |
| Dc | 0.0008 | 0.008 |

| Measurement unit | ‰ | ‰ |
|---|---|---|
| Content | 1.17 | 0.10 |
| Zn/Co | 12.16 | 1 |
| Yield (g) | 42.05 | 42.05 |
| Washings No. | 2 | 2 |

$$‰ (mg/g) = \frac{Ac \times Ps \times fs \times Ds \times 1000}{As \times Pc \times Dc}$$

Ac Sample absorbance
Ps Standard weight
Fs Standard factor
Ds Standard dilution
As Standard absorbance
Pc Sample weight
Dc Sample weight

*Step VI* **Results of biological activity**

| Test | fermentat. of saccharose* after 24 h |
|---|---|
| A,B | negative |

* Saccharose concentration 9.25% (isotonic)

**Example 10: Microorganism modified by incorporation thiamine, Folic acid and cyanocobalamin.**

In this treatment yeast cells have been partially emptied by the hypertonic solution, comprising NaCl and Trisodium citrate bihydrate, as already described. Vitamins were then incorporated by incubation in a vitamins hypotonic solution (hypotonic = 80% isotonicity). The quantity of vitamins loaded into the cells, was measured by HPLC.

Step I) – 168 g of commercially available yeast paste of *Saccharomyces cerevisiae* (corresponding to 56 g of dried yeast) was suspended in 560 g of a NaCl hypertonic solution containing 29.46 g of sodium chloride and 4.94 g of trisodium citrate·$2H_2O$, and kept at 25°C for 16 hours under gentle stirring.

Step II) - The cells were already inactivated by the hypertonic treatment of Step I), and consequently a further inactivation by means of chemico-physical systems was not required.

Step III) – The cellular suspension was centrifuged at 4500 r.p.m. (4596 RCF) for 15 minutes. The centrifugate obtained was washed twice with 560 g of an hypotonic solution (containing: monohydrate tiamin 11.2 g, folic acid 2.8 g and cyanocobalamin 0.28 g). The suspension was resuspended in 560 g of the same solution and kept 16 hours at 25°C under gentle stirring.

*Analytical test of the suspension obtained*

The suspension was then centrifuged and the centrifugates was washed twice with 560 g NaCl isotonic solution (0.9%).

Step IV) - The centrifugate was air-dried at 45°C for 36 hours. After mincing the dried pellets were sifted on a 400μ net.

Step V) – The incorporated vitamins quantities were determined by HPLC after 5N HCl extraction for one hours at 25°C under gentle stirring and after NaOH 0.05N extraction (one hours at 25°C under gentle stirring).

Step VI) - Determination of cell viability

A small part (1 g) of the centrifugates obtained in Step IV) was used to ascertain the vitality of the cells, using the microbiological technique of fermentation of saccharose in a 9.25% isotonic solution and counting of the viable microorganisms with Platecount agar. The results presented in table corresponding to *Step VI* show that no viable cells could be detected after 24 hours of growth, indicating that the microorganisms have been inactivated by the previous treatment in hypertonic solution.

Step VI: Incorporated vitamins as measured by HPLC

HPLC data after HCl extraction

| Batch | Tiamine | Folic Acid | Cyanocobalamin |
|---|---|---|---|
| Ac | 1547.78 | 0.031 | 19.372 |
| Ps | 12.2 | 8.5 | 10.2 |
| Fs | 1.000 | 1.000 | 1.000 |
| Ds | 0.01 | 0.01 | 0.01 |
| As | 2492.881 | 1928.788 | 540.606 |
| Pc | 2000.00 | 2000.00 | 2000.00 |
| Dc | 0.01 | 0.01 | 0.1 |

Incorporated vitamins (HCl extraction)

| Measurement unit | Tiamine | Folic Acid | Cyanocobalamin |
|---|---|---|---|
| Content ‰ (mg/g) | 3.787 | 0.203 | 0.018 |

HPLC data after NaOH extraction

| Batch | Tiamine | Folic Acid | Cyanocobalamin |
|---|---|---|---|
| Ac | 1426.71 | 1225.693 | 14.822 |
| Ps | 12.2 | 8.5 | 10.2 |
| Fs | 1.000 | 1.000 | 1.000 |
| Ds | 0.01 | 0.01 | 0.01 |
| As | 2491.785 | 1956.172 | 540.574 |
| Pc | 2000.00 | 2000.00 | 2000.00 |
| Dc | 0.01 | 0.01 | 0.1 |

Incorporated vitamins (NaOH extraction)

| Measurement unit | Tiamine | Folic Acid | Cyanocobalamin |
|---|---|---|---|
| Content ‰ (mg/g) | 3.493 | 2.663 | 0.014 |

$$‰\ (mg/g) = \frac{Ac \times Ps \times fs \times Ds \times 1000}{As \times Pc \times Dc}$$

Ac: Sample absorbance
Ps: Standard weight
Fs: Standard factor
Ds: Standard dilution
As: Standard absorbance
Pc: Sample weight
Dc: Sample weight

**Example 11: Treatment of the microorganisms with formaldehyde**

Cells obtained from Step III) of each of the Examples 1-10 were treated with formaldehyde.

A suspension was prepared with 10 g of cells per 100 ml of water and was kept under stirring, adding 5 ml of an HCHO solution diluted at 1:10 ml. Stirring was continued for 2 hours, and the cells were washed with water by centrifugation to eliminate the excess HCHO of the non-reacted product.

The treatment carried out on portions of incorporated yeasts obtained from the previous tests enabled an average increase of 15% in the amount of active principle retained inside the cells, by reduction of the leaking amount detectable in the washings for corresponding quantities.

**Example 12: Treatment of the microorganisms with glutaraldehyde**

The cells obtained from Step III) of each of the Examples 1-10 were fixed with glutaraldehyde.

A suspension was prepared with 10 g of treated cells per 100 ml of a NaCl 0.9% solution and was treated with 1 ml of a 1% glutaraldehyde solution, and was left to react for 5 minutes under stirring.

The cells were washed with NaCl 0.9% solution and centrifugated to eliminate the excess non-reacted glutaraldehyde.

The treatment carried out on portions of incorporated yeasts obtained from the previous tests enabled an average increase of 25% in the amount of active principle inside the cells, at the same time reducing the amount detectable in the washings for corresponding quantities.

**Example 13: Microorganism modified by incorporation of α-tocopherol**

**acetate.**

In this treatment yeast cells have been partially emptied by the hypertonic solution, comprising NaCl and Trisodium citrate bihydrate, as already described. α-tocopherol (vitamin E) was then incorporated by incubation in a vitamin hypotonic solution (hypotonic = 80% isotonicity). The quantity of vitamin loaded into the cells, was measured HPLC.

Step I) – 168 g of commercially available yeast paste of *Saccharomyces cerevisiae* (corresponding to 56 g of dried yeast) was suspended in 560 g of a hypertonic solution of NaCl containing 58.45 g/l NaCl and 9.80 g/l trisodium citrate·$2H_2O$) and kept at 25°C for 16 hours under gentle stirring.

Step II) - The cells were already inactivated by the hypertonic treatment of Step I), and consequently a further inactivation by means of chemico-physical systems was not required.

Step III) – The cellular suspension was centrifuged at 4500 r.p.m. (4596 RCF) for 15 minutes. The centrifugate obtained was washed twice with 560 g of an aqueous hypotonic solution containing: α-tocopherol acetate 5.60 g, solubilized in 14g Polyoxyl 35 Castor Oil (NF XVII pag 1966). After centrifugation, the centrifugate was resuspended in 560 g of the same solution and kept 16 hours at 25°C under gentle stirring.

*Analytical test of the suspension obtained*

The suspension was then centrifuged and the centrifugates was washed twice with 560 g NaCl isotonic solution (0.9%) and resupended in the same volume and solution (560 g NaCl isotonic solution 0.9%). 2.8 g formalin 37% was added and the solution was gently stirred for two hours, after which centrifugation was performed. An aliquot of the solution (B), was then washed one more time with a NaCl isotonic solution

Step IV) - The centrifugate was air-dried at 40°C for 36 hours. The dried mass of cells was minced and then sifted on a 400μ net.

Step V) – The incorporated vitamin quantities were determined by HPLC after 5N HCl extraction for one hour at 25°C under gentle stirring.

Step VI) - Determination of cell viability

A small part (1 g) of the centrifugates obtained in Step IV) was used to ascertain

the vitality of the cells, using the microbiological technique of fermentation of saccharose in a 9.25% isotonic solution and counting of the viable microorganisms with Platecount agar. The results presented in table corresponding to *Step VI* show that no viable cells could be detected after 24 hours of growth, indicating that the microorganisms have been inactivated by the previous treatment in hypertonic solution.

Step VI: Incorporated α-tocopherol as measured by HPLC.

Test 43/560

HPLC data

| Batch | A | B |
|---|---|---|
| Ac | 57.601 | 65.224 |
| Ps | 50 | 50 |
| Fs | 1.000 | 1.000 |
| Ds | 0.0025 | 0.0025 |
| As | 642.096 | 642.096 |
| Pc | 1023.6 | 1250.4 |
| Dc | 0.0125 | 0.0125 |

Incorporated α-tocopherol

| Measurement unit | A | B |
|---|---|---|
| Content ‰ (mg/g) | 0.876 | 0.812 |

$$‰\ (mg/g) = \frac{Ac \times Ps \times fs \times Ds \times 1000}{As \times Pc \times Dc}$$

Ac: Sample absorbance

Ps: Standard weight

Fs: Standard factor

Ds: Standard dilution

As: Standard absorbance

Pc: Sample weight

Dc: Sample weight

**Yields of the step:**

| | | |
|---|---|---|
| Drying (g): | A = 42.55 | B = 44.22 |
| Mincing (g): | A = 41.31 | B = 43.67 |
| Loss due to drying: | A = 5.35% | B = 5.54% |

**CLAIMS**

1. Inactivated microorganisms containing one or more of the substances having a pharmacological activity wherein said substances are chosen in the group consisting of: antibiotics, anti-inflammatory, antibacterial, antiviral, antifungal and antiparasitic agents, vaccines, and nutritional substances, provided that said pharmacologically active substances are neither minerals nor enzymes.

2. Inactivated microorganisms according to Claim 1 wherein said antibiotic is oxytetracyclin.

3. Inactivated microorganisms according to Claim 1 wherein said antibacterial is sulfadimethoxine.

4. Inactivated microorganisms according to Claim 1 wherein said nutritional substances are chosen in the group consisting of: vitamins, food integrators, active principles of vegetable origin and nutriceuticals.

5. Inactivated microorganisms according to Claim 4 wherein said vitamins are chosen in the group consisting of: ascorbic acid, cyanocobalamin (vitamin B12), folic acid, thiamine (Vitamin B1), α-tocopherol.

6. Inactivated microorganisms according to Claim 4 wherein said nutriceuticals are bioflavonoids.

7. Inactivated microorganisms according to Claim 6 wherein said bioflavonoids are chosen among: sodium quercetin, catechin, isocatechin, aliphatic polyalcohols, polyphenols, flavans, cyanins, resveratrol, hyperic acid and rutinoids.

8. Inactivated microorganisms according to Claims 1-7, wherein said microorganism is Saccharomyces cerevisiae.

9. Food compositions characterized in that the inactivated microorganisms according to Claims 1-8 are used.

10. Food compositions according to Claim 9 suitable for human and animal use.

11. Food compositions according to Claim 10 wherein said animal are fishes

12. Food compositions according to Claim 11 wherein said fish are in the early stages of growth.

13. Use of one or more inactivated microorganisms according to Claims 1-12, in human or animal alimentation.

14. Use of the microorganisms according to Claims 1-12, as components of

feeding or premixes in zootechnics.

15. Use of the microorganisms according to Claims 10-12 for the feeding of fish.

16. Process for the preparation of inactivated microorganisms containing one or more soluble and/or solubilizable substances having pharmacological activity and/or nutritional substances having pharmacological activity, according to Claims 1-12, comprising the following steps:

i) drawing out the endocellular mass of a suitable microorganism by means of hypertonic treatment, separation of the drawn out endocellular mass and recovery of the empty microorganisms;

ii) optional inactivation of the microorganism obtained in Step i) chemically or physically, leaving the external membrane of the microorganism unaltered; and

iii) intracellular loading of one or more soluble and/or solubilizable substances having pharmacological activity and/or nutritional substances having pharmacological activity, into the inactivated microorganism obtained in Step i) or Step ii), is obtained by means of hypo- and/or iso-tonic treatment.

17. Process for the preparation of inactivated microorganisms according to Claim 16, characterized in that:

in Step i) the drawing out of the endocellular mass is obtained by incubation in a hypertonic solution of the same pharmacologically active substance to be loaded into the microorganism;

in Step iii) said pharmacologically active substance is already present in the solution and is loaded into the microorganism with a change of the osmolarity due to dilution of the solution to hypo- and/or isotonicity.

18. Process according to Claims 16 and 17, characterized in that Step iii) is followed by treatment of the microorganisms with a fixative or a disinfectant agent

19. Process for the preparation of inactivated microorganisms described in Claims 1-12, according to the following steps:

1) the microorganism is inactivated by thermal treatment, at 60-65°C for 30-120 min;

2) the inactivated cells of the microorganism are resuspended in an isotonic medium comprising the active principle to be incorporated;

3) the suspension is left under stirring for 48-72 hours;

4) the suspension is centrifuged;

5) optional buffering and/or fixation is carried out using formalin and/or glutaraldehyde.

20. Process according to Claims 16 and 17, characterized in that the hypertonic treatment in step i) is obtained by incubation of a hypertonic solution comprising:

- NaCl in concentrations higher than 0.2 M;
- optionally sodium citrate 0.03 - 0.1 M.

21. Process according to Claims 16 and 17, in which said hypotonic treatment in step iii) is obtained by means of a hypotonic solution comprising:

- NaCl in concentrations lower than 0.12 M;
- optionally sodium citrate in concentrations lower than 0.025 M.

22. Process according to Claim 16, in which the isotonic treatment in step iii) is performed by a 0.9% NaCl isotonic solution, optionally comprising sodium citrate 0.025 M.

23. Process according to Claim 16, in which

- said hypertonic solution is NaCl 1.0 M and sodium citrate 0.05 M;
- said hypotonic solution is NaCl 0.05 M and sodium citrate 0.005 M

24. Process according to Claim 16, in which

- said hypertonic solution is NaCl 1.0 M and sodium citrate 0.05 M;
- said isotonic solution is NaCl 0.9% and sodium citrate 0.025 M.

# UNITED STATES

| UTILITY PATENT APPLICATION<br>DECLARATION AND POWER OF ATTORNEY – ORIGINAL APPLICATION | ATTORNEY'S DOCKET NO.<br>205,360 |
|---|---|

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below next to my name:
I verily believe I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the invention entitled

(1) TITLE OF INVENTION: (1) INACTIVATED MICROORGANISMS COMPRISING SUBSTANCES HAVING PHARMACOLOGICAL ACTIVITY

the specification of which

(2) CHECK APPROPRIATE BOX:
(2) [ ] is attached hereto.
[x] was filed on April 20, 2000 as Application No. PCT/EP00/03586
and was amended on 27.04.2001 (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.
I acknowledge my duty to disclose information of which I am aware which is material to the patentability of this application under 37 CFR 1.56(a): the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months prior to this application; and as to applications for patents or inventor's certificate on the invention filed in any country foreign to the United States prior to this application by me or my legal representatives or assigns.

(3) CHECK APPROPRIATE BOX:
(3) [ ] no such applications have been filed, or
[x] such application(s) have been filed as follows:

(4) COMPLETE DATA INDICATED IF APPLICABLE

| EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED WITHIN 12 MONTHS PRIOR TO THIS APPLICATION | | | | |
|---|---|---|---|---|
| Country | Application Number | Date of Filing (day, month, year) | Date of Issue (day, month, year) | Priority Claimed Under 35 USC 119 |
| (4) ITALY | MI99A000842 | 22 APRIL 1999 | | X Yes No |
| | | | | Yes No |
| | | | | Yes No |
| ALL FOREIGN APPLICATIONS, IF ANY, FILED MORE THAN 12 MONTHS PRIOR TO THIS APPLICATION | | | | |
| (4) | | | | |
| | | | | |

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112. I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

(5) COMPLETE DATA INDICATED IF APPLICABLE

(5) _____ (Application Serial No.) (Filing date) (Status: patented, pending, abandoned)

(5) _____ (Application Serial No.) (Filing date) (Status: patented, pending, abandoned)

Power of Attorney: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(6) DETAILS REQUIRED FOR EACH INVENTOR

| Full Name of Sole or First Inventor | Inventor's Signature | Date |
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| Post Office Address | | |
| Full Name of Fifth Joint Inventor, If Any | Inventor's Signature | Date |
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| Full Name of Sixth Joint Inventor, If Any | Inventor's Signature | Date |
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